Loss of ecto-5′ nucleotidase from porcine endothelial cells after exposure to human blood: Implications for xenotransplantation

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Abstract

The endothelial cell surface expression of ecto-5′-nucleotidase (E5′N, CD73) is thought to be essential for the extracellular formation of cytoprotective, anti-thrombotic and immunosuppressive adenosine. Decreased E5′N activity may play a role in xenograft acute vascular rejection, preventing accommodation and tolerance mechanisms. We investigated the extent of changes in E5′N activity and other enzymes of purine metabolism in porcine hearts or endothelial cells when exposed to human blood or plasma and studied the role of humoral immunity in this context.

Pig hearts, wild type (WT, n = 6) and transgenic (T, n = 5) for human decay accelerating factor (hDAF), were perfused ex vivo with fresh human blood for 4 h. Pig aortic endothelial cells (PAEC) were exposed for 3 h to autologous porcine plasma (PP), normal (NHP) or heat inactivated human plasma (HHP), with and without C1-inhibitor. Enzyme activities were measured in heart or endothelial cell homogenates with an HPLC based procedure.

The baseline activity of E5′N in WT and T porcine hearts were 6.60 ± 0.33 nmol/min/mg protein and 8.54 ± 2.10 nmol/min/mg protein respectively (P < 0.01). Ex vivo perfusion of pig hearts with fresh human blood for 4 h resulted in a decrease in E5′N activity to 4.01 ± 0.32 and 4.52 ± 0.52 nmol/min/mg protein (P < 0.001) in WT and T hearts respectively, despite attenuation of hyperacute rejection in transgenic pigs. The initial PAEC activity of E5′N was 9.10 ± 1.40 nmol/min/mg protein. Activity decreased to 6.76 ± 0.57 and 4.58 ± 0.47 nmol/min/mg protein (P < 0.01) after 3 h exposure of HHP and NHP respectively (P < 0.05), whereas it remained unchanged at 9.62 ± 0.88 nmol/min/mg protein when incubated with PP controls. C1-inhibitor partially preserved E5′N activity, similar to the effect of HHP. Adenosine deaminase, adenosine kinase and AMP deaminase (other enzymes of purine metabolism) showed a downward trend in activity, but none were statistically significant.

We demonstrate a specific decrease in E5′N activity in pig hearts following exposure to human blood which impairs adenosine production resulting in a loss of a cytoprotective phenotype, contributing to xenograft rejection. This effect is triggered by human humoral immune responses, and complement contributes but does not fully mediate E5′N depletion.

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Keywords: Ecto-5′-nucleotidase; Adenosine; Xenotransplantation; Complement; Cytoprotection

Abbreviations: E5′N or CD73, ecto-5′-nucleotidase; hDAF, human decay accelerating factor; EC, endothelial cell; NTPDase 1 or CD39, nucleoside triphosphate diphosphohydrolase

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1. Introduction

Human organ failure is the foremost cause of death and disability. Human organ shortages are a vexing challenge, leading scientists to explore animal organs as alternative sources. However, acute vascular rejection is a major obstacle in the clinical application of xenotransplantation. This process is characterised by endothelial cell (EC) activation, antibody-mediated damage within the graft, infiltration by host monocytes and NK cells and platelet sequestration with vascular thrombosis secondary to the loss of protective thromboregulatory elements from the vascular lumen [1–4]. However, cell bioenergetics and metabolic sequelae following xenogeneic humoral immune responses remain ill-defined.

The production of adenosine may have an important cytoprotective role in xenograft acute vascular rejection. Adenosine can be formed as an intracellular process or extracellularly from the catabolic breakdown of pro-inflammatory and pro-aggregatory nucleotides (ADP and AMP) by a glycosyl phosphoinositol (GPI) extracellular membrane-linked enzyme, ecto-5′-nucleotidase (E5′N, CD73, EC 3.1.3.5.) [5]. Adenosine and purinergic signalling elicit a broad spectrum of cytoprotective, immunosuppressive and platelet inhibitory effects that has been a research focus for several decades [6–8].

Nucleotide metabolic changes have been undetermined in the context of xenogeneic tolerance (immune non-responsiveness) [9,10] and/or accommodation (acquired resistance of an organ to humoral injury) [11,12]. Xenoreactive antibodies and non-αGal specific antibodies can give rise to acute vascular rejection by alternative mechanisms [13–15]. Optimizing tolerance and accommodation strategies in xenotransplantation has recently been identified in a process leading to accommodation which occurs when xenoreactive antibodies bind to αGal epitopes on Adenosine 2a (A2a) receptors, promoting neo-expression of Bel-xL (a cytoprotective protein) in endothelial cells [16]. Consequently, the regulation of E5′N expression and activity may play an important role in xenotransplantation. Decreased E5′N activity may impair adenosine production, promote acute vascular rejection, thrombo-dysregulation and decrease the neo-expression of cytoprotective proteins. This results in decreased xenograft accommodation, increased cytotoxic lymphocyte effects, neutrophil infiltration, free-radical injury and thrombus formation [17–20].

In the present study, we investigate the activities of E5′N and other enzymes of adenosine metabolism by exposing porcine hearts or endothelial cells to human blood or plasma. Furthermore, by using transgenic hearts over-expressing human decay accelerating factor (hDAF), a complement regulating protein, and heat inactivated human plasma with or without C1-inhibitor, we aimed to clarify the role of humoral immunity in the context of nucleotide metabolism and xenograft rejection. These results provide evidence that changes in nucleotide surface enzymes such as E5′N may play a significant role in xenograft rejection.

2. Materials and methods

2.1. Materials

Reagents for enzyme analyses and HPLC grade reagents were obtained from Merek/BDH. (8,14C)-adenosine, used to assess the activity of adenosine kinase, with a specific radioactivity of 50 μCi/μmol, was obtained from ICN (Hants, UK). Collagenase, cell culture media, antibiotics, foetal calf serum, growth factors, AMP, erythro-9-(hydroxy-3-onyl)adenine (EHNA, adenosine deaminase inhibitor), 5′-amino-5′-deoxyadenosine, ATP, phosphocreatine, creatine kinase, adenylyl kinase, adenosine and all other chemicals were purchased from Sigma.

2.2. Heart perfusion

The perfusion system included a centrifugal pump (Medtronics), blood reservoir, thermostatically controlled paediatric blood oxygenator with outflow connected to aortic cannula, water jacketed heart compartment, haemofilter and a thermostated circulator maintaining the temperature of the blood oxygenator and heart compartment at 37 °C. Wild type (WT, n = 6) and transgenic (T, n = 5) pigs of either sex weighing 70–120 kg were anesthetized and intubated. The chest was then opened and aorta cannulated and clamped. Hearts were then infused with cold St. Thomas’ Hospital No.1 cardioplegic solution before removal from the chest and connected to the perfusion apparatus. The system was filled with 1.2 l of freshly collected heparinized human blood pooled from three ABO-matched donors. Initial flow was adjusted to obtain a perfusion pressure of 60 mm Hg. This was achieved with a blood flow rate within the range of 0.4–0.6 l/min in all experiments. This flow setting was maintained throughout perfusion. All perfusion experiments were carried out for 4 h. Biopsy specimens for enzyme assays were collected using Travenol needles at the beginning and at the end of perfusion. Specimens were immediately placed and stored in liquid nitrogen until assay.

2.3. Cell culture

Pig aortic endothelial cells (PAEC) were prepared from porcine aortas as described in detail previously [21]. Briefly, cells were plated on gelatine coated 25 cm² plastic culture flasks with medium M 199, 10% heat-inactivated newborn calf serum, 10% heat-inactivated fetal calf serum, supplemented with 2 mM L-glutamine, 150 U/ml penicillin, 150 U/ml streptomycin, 50 μg/ml gentamycin and 2.5 μg/ml amphotericin B. Endothelial cells had their typical cobblestone appearance in phase contrast microscopy.
2.4. Exposure of endothelial cells to human and porcine plasma with and without complement inhibitor

Human and porcine blood was collected in 7.5 ml endotoxin-free lithium–heparin (15 IU/ml, final concentration) coated glass tubes (Beckman Coulter, UK). Tubes were immediately centrifuged at 3500 × g for 5 min and plasma collected. One portion of plasma was heat inactivated at 56 °C for 1 h. PAEC were grown to confluence. The cells were then washed in serum-free M199 media and incubated in media with 50% fresh human (NHP) or porcine plasma (PP) or 50% heat-inactivated human plasma (HHP). PAEC were incubated with human or porcine plasma for 0 or 180 min (for 0, 15, 60, or 180 min in preliminary experiments) at 37 °C. E5′N, other enzymes of nucleotide metabolism and lactate dehydrogenase were assessed in cell homogenates (see below). In some experiments, human C1-inhibitor (Aventis-Behring, UK) was added during incubation with porcine and human plasma for up to 180 min at concentrations of 4 mg/ml (data not shown) and 8 mg/ml as has been described by Solvik et al. [22]. E5′N and other enzymes of nucleotide metabolism were assessed in cell homogenates as indicated below.

2.5. Enzyme assays

The adenosine metabolizing enzymes were assayed in homogenates of heart biopsies collected before and at the end of perfusion as we previously described [23]. PAEC homogenates for enzyme assay were prepared from confluent endothelial cells (as above). Supernatants of experimental incubation samples were aspirated and cells washed three times with phosphate-buffered saline (PBS). A homogenisation buffer containing 150 mM KCl, 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol (pH 7.0) plus 0.1% Triton X-100 (0.1 ml) (cell lysis and to solubilise membrane) was added in three portions to maximise cell recovery. Endothelial cell homogenates were diluted with an incubation buffer before assay. To determine E5′N, adenosine deaminase, adenosine kinase and purine nucleoside phosphorylase, cell homogenates were diluted with the appropriate incubation buffer [23]. The substrate concentration for E5′N was 0.2 mM AMP in the presence of 5 μM EHNA. This assay is optimal for the measurement of E5′N and distinguishes from the activity of cytosolic S′-nucleotidases that require activators (e.g. ADP) and higher substrate concentration. EHNA stopped adenosine deaminase converting adenosine into inosine. Blocking the downstream pathway was essential to accurately measure the formation of adenosine by HPLC. For the measurement of ADA, 1 mM adenosine was used and for PNP, and 1 mM inosine was used as the substrate. For the assay of adenosine kinase, the substrate solution included 5 μM (8-14C)-adenosine with a specific radioactivity of 50 μCi/μmol, which was added together with 4 mM ATP, 5 μM EHNA, 8 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase and 0.03 mg/ml adenylyl kinase. All enzyme reactions were initiated by the addition of 20 μl of diluted cell homogenate to 20 μl of substrate followed by incubation at 37 °C and the reaction was stopped with the addition of 20 μl of 1.3 M HClO4. Each sample was neutralised with ~5 μl of 3 M K2HPO4, before the analysis of substrate conversion into products by reversed-phase HPLC [24]. Protein concentration was determined using the Bradford method [25]. Lactate Dehydrogenase (LDH) was assayed in the cell homogenates by measuring the rate of production of NAD+ at 340 nm with pyruvate as substrate at 30 °C [26].

2.6. Statistical analysis

All values are presented as the mean ± standard error (S.E.). In vitro cultured cell experiments were repeated 4–6 times, with measurement performed in triplicate, each with its own control during every experiment. Comparisons of measurements in cultured cells experiments at different time points have been performed using paired Student’s t-test. Comparisons between groups at the same time point have been performed using unpaired Student t-test or one way analysis of variance (ANOVA). For the analysis of enzyme activities during ex vivo perfusion, due to significant difference of variances, non-parametric Mann–Whitney U-test was used. Statistical significance was defined as P less than 0.05 (two-tailed).

3. Results

The activity of E5′N was 6.60 ± 0.33 nmol/min/mg protein in wild type (WT) pig hearts (n = 6) and 8.54 ± 2.10 nmol/min/mg protein in transgenic (T) pigs (n = 5) over-expressing hDAF at the start of perfusion with fresh human blood (P < 0.01) (Fig. 1). Ex vivo perfusion of pig hearts with fresh human blood for 4 h resulted in a decrease in E5′N activity to 4.01 ± 0.32 in WT pig hearts and to 4.52 ± 0.52 nmol/min/mg protein in T pig hearts respectively (P < 0.001). This decrease in the activity of E5′N occurred despite the attenuation of
immune, functional and structural changes typical for hyper-
acute rejection in T hearts (Smolenksi et al., in preparation).
No significant changes in the adenosine deaminase (ADA),
adenosine kinase (AK) or AMP deaminase activities were
observed (Table 1), although the trend for the enzyme
activities was downward; while purine nucleoside phosphor-
ylase (PNP) activity significantly increased in WT hearts after
4 h of perfusion ($P<0.01$).

The initial pig aortic endothelial activity of E5’N was
9.10 ± 1.40, 9.62 ± 1.56 and 9.15 ± 1.87 nmol/min/mg protein
in the control pig plasma (PP), heat inactivated human
plasma (HHP) and normal human plasma (NHP) groups
respectively (Fig. 2). Activity decreased to 4.58 ± 0.47 and
6.76 ± 0.57 nmol/min/mg protein after 3 h exposure of NHP
and HHP groups respectively, while it remained constant at
9.62 ± 0.88 nmol/min/mg protein in PP controls. Incubation
with C1-inhibitor added to NHP indicated partial attenu-
ation of E5’N activity decrease, to the same level as in
HHP. Separate experiments indicated that E5’N activity
remained constant during 3 h incubation with Hanks
balanced salt solution (results not shown). There were no
significant changes in AK or ADA activities during
incubation in any groups, although there was a downward
trend in activity. A slight significant increase in PNP
activity was observed in cells incubated with NHP (Table
2). The activity of lactate dehydrogenase (LDH) was
3.75 ± 0.40 nmol/min/mg protein initially and remained at
100%, 100%, 96% in NHP, HHP and PP control treated
cells after 3 h of incubation.

4. Discussion

We demonstrate a novel potential mechanism of xeno-
graft dysfunction that may involve a reduction in the
capacity of the endothelium to convert pro-inflammatory
and pro-aggregatory extracellular nucleotides into adenosine
due to a specific decrease of E5’N activity. The decrease in
E5’N activity occurs rapidly following contact with human
blood or plasma. Complement depletion (heat inactivation)
of human plasma or C1-inhibition partially attenuated E5’N
activity decrease while the over-expression of human decay
accelerating factor (hDAF) in transgenic hearts was unable
to block the specific decrease of E5’N activity. These results
suggest that complement inhibition is not sufficient to fully
protect porcine organs from injury from human plasma and
highlights other factors that may lead to a depletion of E5’N.
Understanding the changes in E5’N activity in allo- [27] and
xenotransplant settings may lead to a better mechanistic

Table 1
Activities of adenosine deaminase, purine nucleoside phosphorylase and
adenosine kinase in wild type (WT) or transgenic (T) pig hearts perfused
with human blood for 240 min

<table>
<thead>
<tr>
<th></th>
<th>Wild type pigs</th>
<th>Transgenic pigs</th>
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<tbody>
<tr>
<td>Purine nucleoside phosphorylase (nmol/min/mg protein)</td>
<td></td>
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<tr>
<td>0 min</td>
<td>45 ± 16</td>
<td>58 ± 32</td>
</tr>
<tr>
<td>240 min</td>
<td>100 ± 26*</td>
<td>86 ± 38</td>
</tr>
<tr>
<td>Adenosine deaminase (nmol/min/mg protein)</td>
<td></td>
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</tr>
<tr>
<td>0 min</td>
<td>9.31 ± 1.12</td>
<td>7.90 ± 0.18</td>
</tr>
<tr>
<td>240 min</td>
<td>6.11 ± 0.56</td>
<td>5.85 ± 1.10</td>
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<tr>
<td>Adenosine kinase (nmol/min/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.15 ± 0.12</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>240 min</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
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<tr>
<td>AMP deaminase (nmol/min/mg protein)</td>
<td></td>
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<tr>
<td>0 min</td>
<td>13.6 ± 2.6</td>
<td>13.6 ± 1.5</td>
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<tr>
<td>240 min</td>
<td>9.9 ± 1.1</td>
<td>11.4 ± 2.4</td>
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</tbody>
</table>

Values represent the means ± S.E., n = 6 for WT; n = 5 for T. *P < 0.01 vs. 0 min.

Table 2
Activities of adenosine deaminase, purine nucleoside phosphorylase, adenosine kinase and lactate dehydrogenase in pig aortic endothelial cells
incubated for 0 or 180 min with control porcine plasma (PP), normal human plasma (NHP) or heat inactivated human plasma (HHP), and culture media
(data not shown)

<table>
<thead>
<tr>
<th></th>
<th>Control porcine plasma</th>
<th>Heat inactivated human plasma</th>
<th>Normal human plasma</th>
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<tbody>
<tr>
<td>Purine nucleoside phosphorylase (nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>35.3 ± 3.3</td>
<td>31.0 ± 3.4</td>
<td>27.6 ± 1.6</td>
</tr>
<tr>
<td>180 min</td>
<td>31.6 ± 2.3</td>
<td>28.0 ± 2.4</td>
<td>33.2 ± 1.8*</td>
</tr>
<tr>
<td>Adenosine deaminase (nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.37 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>0.32 ± 0.09</td>
</tr>
<tr>
<td>180 min</td>
<td>0.22 ± 0.05</td>
<td>0.20 ± 0.03</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>Adenosine kinase (nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.48 ± 0.06</td>
<td>0.58 ± 0.06</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>180 min</td>
<td>0.40 ± 0.03</td>
<td>0.57 ± 0.17</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>Lactate dehydrogenase (nmol/min/mg protein)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0 min</td>
<td>3.75 ± 0.28</td>
<td>3.84 ± 0.40</td>
<td>3.70 ± 0.40</td>
</tr>
<tr>
<td>180 min</td>
<td>3.85 ± 0.21</td>
<td>3.91 ± 0.38</td>
<td>3.76 ± 0.69</td>
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</table>

Values are means ± S.E., n = 4–6; measurements were performed in triplicate during each experiment. *P < 0.01 vs. 0 min.
understanding of acute vascular rejection, thromboregulation and xenograft cytoprotection.

It has long been known that hypoxia, ischaemia and inflammation all stimulate local adenosine production [8,28,29]. Because the endothelium is a barrier to adenosine, adenosine formed within the lumen of blood vessels may be derived in large part from platelets (ATP, ADP) and endothelium. Acute vascular rejection results in endothelial damage, activation of platelets (ADP exerts pro-aggregatory effect via cell surface P2Y receptors), acute vascular thrombosis and, subsequently, organ ischaemia. Hypoxia causes the release of intracellular adenine nucleotides and subsequent degradation upregulates (via hypoxia-inducible factor 1α) [30,31] the adenine nucleotide metabolizing ecto-nucleotidases such as E5’N and nucleoside triphosphate diphosphohydrolase NTPDase 1 (CD39, ecto-ATPase, EC 3.6.1.5), an enzyme found upstream of E5’N (main substrates are ATP and ADP) [32]. This eventually leads to increased extracellular adenosine that activates anti-aggregatory A2a receptors. NTPDase is therefore a major thromboregulatory element associated with the vascular endothelium and has a critical role for the regulation of platelet thrombosis and blood fluidity [33] by modulating purinergic receptor signalling [34]. For example, the infusion of a soluble plant NTPDase or apyrase prolongs xenograft survival and recent studies have shown that adenoviral induced over-expression of CD39 within mouse-to-rat cardiac xenografts attenuates xenograft malfunction, presumably by overcoming the procoagulant phenotype [35]. A reduction of NTPDase activity has also been demonstrated during endothelial activation and is considered to be an important mechanism of xenotransplant dysfunction [36]. Although E5’N activity or expression changes have not been investigated so far in the context of xenotransplantation, there is extensive evidence that endothelial E5’N activity plays a major role in the regulation of platelet aggregation and inflammatory responses [37,38]. An increased expression of E5’N has been identified as an important mechanism of action of anti-inflammatory drugs such as methotrexate and sulfasalazine [39]. Recent studies demonstrate the crucial role of endothelial E5’N expression in the regulation of platelet aggregation [40] and the augmentation of vascular inflammatory responses, featuring its important role on coronary vascular endothelium [41]. Our observation that E5’N decreases in pig hearts and endothelium following exposure of human blood or plasma may thus indicate mechanisms which could favour a pro-inflammatory and pro-aggregatory milieu which exacerbates xenotransplant dysfunction either by failing to produce enough extracellular adenosine to stimulate A2a receptors (loss of cytoprotection) or may exacerbate platelet dysregulation (acute vascular thrombosis).

Interpretation of the new data suggests that E5’N activity decrease is due to thermostable antibodies. A direct binding of cross-reacting α-Gal-specific antibodies that are present in human plasma may be involved, similar to α-Gal antibodies that cross-react with epitopes on A2a receptors [42]. It is possible that such antibodies may cross-react or block the active site of E5’N, which is in close vicinity to A2a receptors found on the endothelial cell surface [43]. Although our data indicates a partial protection of E5’N activity by heat inactivation of plasma, more so than C1-inhibition of the complement cascade, it did not completely prevent a decrease of E5’N activity, suggesting that thermostable antibodies, proximal to the complement cascade, contribute to E5’N inactivation. However, the precise role, character and subclass of thermostable antibodies (particularly IgG and IgM α-Gal-specific antibodies) present in heat inactivated plasma and the relationship with E5’N and purinergic signalling should be investigated in further studies to clarify this assumption. One complicating factor that we were unable to avoid was the addition of heparin during blood collection at relatively high dose (15 IU/ml). This concentration is at the lower threshold for the inhibition of complement activation [44,45]. To eliminate this variable in future complement-sensitive studies, recombinant hirudin analogues like lepirudin which does not interfere with complement activation could be used as standard anticoagulation [46]. The involvement of binding of elements proximal to C3 is further supported by our data which shows that over-expression of human decay accelerating factor (hDAF) (a C3 convertase) in transgenic pig hearts was unable to block the loss of E5’N activity. Other authors have shown that in an ex vivo perfusion lung xenotransplant model complement inhibition with C1-inhibition or hDAF is not effective in preventing the sequelae of humoral immunity or acute vascular rejection [47]. Interestingly, the initial activity of E5’N in hDAF overexpressing hearts was higher than wild type E5’N activity (P<0.01), which suggests that hDAF may regulate E5’N expression or modulate E5’N activity.

Another possibility that may lead to a specific depletion of E5’N activity is the disruption of the membrane structures that harbour E5’N activity. It is known that adenosine receptors are concentrated in caveolin enriched plasma membrane microdomains referred to as caveolae [48]. E5’N, like CD39, is preferentially targeted to these membrane microdomains which are involved in signalling (eNOS, G-proteins, receptors) and are targeted to the caveolae undergoing post-translational modifications (e.g., palmitoylation). Furthermore, the presence of ecto-nucleotidases in caveolae supports their participation in signalling events [49]. A further possibility could be that factors that enhance E5’N activity such as IFN-α and other cytokines may be depleted, supporting a downregulation of E5’N [50]. A salient finding that we found was a specific decrease in E5’N activity. The activity of cytosolic 5’-nucleotidase in tissue and cell homogenates can be disregarded in part as assay conditions were optimal for E5’N in terms of extracellular environment, lack of essential activators for cytosolic 5’-nucleotidases and by the use of suboptimal substrate concentrations normally used to measure cytosolic...
enzymes [51]. Our unpublished data on E5N transfected PAEC indicate that E5N activity in cell homogenates correlates with an intact cell’s ability to degrade extracellular AMP. The possibility that this effect represents a consequence of non-specific endothelial cell damage can be excluded since we measured the activity in relation to total cellular protein content which compensated for cell lysis induced by complement. Taken together, this data and the unchanged lactate dehydrogenase specific activity in all in vitro groups before and after incubation provides evidence that E5N activity decrease is specific.

Adenosine deaminase is an enzyme that is partially membrane bound but also found in the cytosol, while adenosine kinase activity is solely cytosolic [52]. In our experiments, a downward trend of adenosine deaminase activity was observed ($P = \text{NS}$) and can be attributed to the loss of the extracellular form, but proving this requires further studies. Ex vivo perfusion experiments in wild type and transgenic hearts show a relative two-fold increase in purine nucleoside phosphorylase. Furthermore, we observed a smaller increase ($P < 0.01$) in purine nucleoside phosphorylase activity in vitro. Purine nucleoside phosphorylase normally degrades inosine or guanosine, but may be involved in the catabolism of deoxyguanosine especially in lymphoid cells [53]. It is also important to note that normal human erythrocyte activity of purine nucleoside phosphorylase is almost 25 times higher than baseline activity of this enzyme in pig hearts [54]. The increase in purine nucleoside phosphorylase found in ex-vivo experiments can in part be explained by sequestered human erythrocytes within the porcine hearts or actively dividing human T cells found infiltrating into porcine hearts. However, to explain the in vitro findings, further investigation of experimental conditions need to be done which may reveal possible responses to increased levels of deoxyguanosine, dGTP and nucleosidase activities following endothelial cell activation or apoptosis.

Although ex-vivo perfusion with autologous porcine blood was not performed to evaluate the effect of the perfusion itself on E5N activity, our previous experience with purine enzyme activities measurements in a small animal perfusion system indicate that any changes are unlikely [55]. Furthermore, lack of changes of in vitro E5N activity during incubation with Hanks solution or porcine plasma render the possibility of any non-specific effects unlikely.

This study provides evidence that metabolic pathways and humoral immunity intersect and that changes in metabolic pathways may play a role in xenograft dysfunction. Understanding the role of nucleotide metabolism and purinergic signalling in xenograft rejection may lead to therapeutic avenues for improving the outcome of future xenografts.

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